REMARKS

Claims 6, 9 and 10 are all the claims pending in the application.

In the present Amendment, claim 6 is amended to recite a process for producing a natural cheese, which comprises (1) incubating a lactic acid bacteria starter comprising a lactic acid bacteria with culture medium containing milk component wherein yeast extract is added; (2) adding the incubated lactic acid bacteria starter to a raw milk; (3) forming a curd from the raw milk mixed with the lactic acid bacteria starter; (4) removing whey from thus formed curd; and (5) forming pressed pieces of the curd including molding and pressing the curd, wherein the process further comprises adding additional yeast extract to the raw milk at the same time or after adding the lactic acid bacteria starter to the raw milk in step (2), and before formation of the curd in step (3); and incubating the curd obtained in the above (5), at 20 to 35°C for 16 to 26 hours to produce the natural cheese, wherein the incubation of the curd is carried out without cooling the curd after molding and pressing, wherein ... .

Support for the amendment to claim 6 can be found in the specification, for example, at pages 23-24, Example 3. In Example 3, *L. gasseri* OLL 2716 was inoculated at a ratio of 1% into a 10% skim milk medium containing 0.1% **yeast extract**. Then *L. gasseri* was cultured at 37°C for 24 hours, thereby giving bulk starters. Subsequently, 20 kg of partially skim milk (SNF 8.5%, fat 3%), which had been sterilized at 73°C for 15 seconds, was adjusted to 32°C and inoculated with 1% of the *L. gasseri* bulk starter. Next, 20 g of **yeast extract was further added**. Then cheese curd was produced by a conventional method, pressed and incubated in a mold in a room at a room temperature of 25°C for 24 hours.

The process of making cheese according to Example 3 includes first adding an yeast extract to a 10% skim milk to make bulk starter containing *L. gasseri*, **and** then adding additional yeast extract after adding the lactic acid bacteria bulk starter to raw milk and before formation of the curd in step (2). That is, the yeast exact are added to the process twice.

No new matter has been introduced. Entry of the Amendment is respectfully requested.

I. Claim Rejections under 35 U.S.C. § 103

Claims 6-7, and 9-10 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Gardiner et at. (1998, Development of a probiotic cheddar cheese containing human -derived Lactobacillus paracasei strains; hereinafter "Gardiner") in view of DE 1955833 (hereinafter "R2") and Kimura et al. (EP 1 112 692 Al, hereinafter "Kimura").

Claims 6-7 and 9-10 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over R2, Kimura et al. (EP 1 112 692 Al, hereinafter "Kimura"), further in view of Germond et al. (WO 0188150, hereinafter "Germond").

Applicants traverse the above rejections for at least the reasons presented in the previously submitted Amendments and Responses, which are not be repeated herein.

Further, in the Advisory Action of March 16, 2010, the Examiner provided comments on the Declaration by Mr. Matsuo submitted on February 11, 2010 together with the Amendment under 37 C.F.R. § 1.116.

In particular, regarding the claimed feature of "viable cell count overtime," the Examiner asserted that Gardiner et at. (1998, referred to by the Examiner as "R1", hereinafter as "Gardiner") discloses bacterial counts of their product. According to the Examiner, Gardiner

discloses that after 8.5 months at 8 °C, the count is in the ten to hundred million per gram ranges. *See* the Advisory Action, at page 3, paragraph c.

Gardiner relates to preparation of cheddar cheese containing live cultures of probiotic *Lactobacilli*. Gardiner is said to disclose that cheese made with *L. paracasei* contained high levels of these probiotic strains after 8 months of ripening with final counts of 10^7-10^8 CFU/g cheese (page 2195, Col. 1, last two lines to Col. 2, first two lines).

It is the Examiner's position that since Gardiner discloses that the probiotic *L. paracasei* strains incorporated into cheddar cheese grow and proliferate to high cell numbers in cheese overtime, it would have been obvious for ordinary skilled in the art to consider Cheddar cheese as an effective vehicle for delivery of *Lactobacillus gasseri* strains to the consumer.

Applicants respectfully disagree. There is no reasonable scientific basis to assume all *Lactobacillus* strains have same growth rate and survival rate overtime when incorporated into cheese. It is not reasonable to assume that *L. paracasei* (disclosed in Gardiner) and *Lactobacillus gasseri* strains would be interchangeable to produce predictable results with reasonable expectation of success.

Indeed, *Lactobacillus paracasei* (used in Gardiner) is well known to be resistant in environments and thus has a greater viability than other lactobacillus species including *L. gasseri, L. casei, L. acidophilus, L. rhamnosus*, and others. Applicants submit herewith a copy of a publication entitled "Viability of commercial probiotic cultures (*L. acidophilus, Bifidobacterium* sp., *L. casei, L. paracasei and L. rhamnosus*) in cheddar cheese." In the publication, Applicants recognize that the publication does not discuss or compare directly *L.*

gasseri and *L. paracasei*. However, *L. gasseri* was used to be classified in *L. acidphilus* and they have similar genetical and morphological characterizations. Therefore, one ordinary skilled in the art would understand that *L. gasseri* would show similar viability to *L. acidphilus*. As shown in Fig. 2 in comparison with Fig. 3, the bacterial count of *L. acidphilus* in cheese (Fig. 2) decreased earlier than *L. paracasei* (Fig. 3). The survive rate of *L. acidphilus* (shown in Fig. 2) in cheese after 30 weeks is well below the level of *L. paracasei (*shown in Fig. 3). That is, the data shows that it is unreasonable to assume and conclude that all species of *Latobacillus* would show a same or similar survival pattern as *L. paracasei*, and that *L. gasseri* would show a similar viability to *L. paracasei* in cheese after overtime. Also, in view of the scientific fact that *L. gasseri* has similar morphological and genetical characterizations to *L. acidphilus*, it can easily be understood that *L. gasseri* would show a lower viability than *L. parasite* in a cheese over the time, if manufactured under same conditions.

In addition, claim 6, as amended, recites in part, a process for producing a natural cheese, which comprises (1) incubating a lactic acid bacteria starter comprising a lactic acid bacteria with culture medium containing milk component wherein yeast extract is added; (2) adding the incubated lactic acid bacteria starter to a raw milk; (3) forming a curd from the raw milk mixed with the lactic acid bacteria starter; (4) removing whey from thus formed curd; and (5) forming pressed pieces of the curd including molding and pressing the curd, wherein the process further comprises adding additional yeast extract to the raw milk at the same time or after adding the lactic acid bacteria starter to the raw milk in step (2), and before formation of the curd in step (3); and incubating the curd obtained in the above (5), at 20 to 35°C for 16 to 26

hours to produce the natural cheese, wherein the incubation of the curd is carried out without cooling the curd after molding and pressing.

R2 is cited by the Examiner as assertedly disclosing a process where cheese of all types with improved storage life, higher yield and improved aroma are obtained by replacing or supplementing conventional cheese cultures with Bifidus bacteria and preferably *adding growth activators such as yeast extract* to the milk (Abstract).

However, R2 only discloses adding yeast extract as growth activators; R2 does not disclose or recognize the addition of an yeast extract before formation of the curd and after incubation of the lactic acid bacteria starter so as to allow L. gasseri grow and survive in cheese dominantly over lactic acid bacteria for cheese. Further, quite clearly, R2 does not disclose or teach that the yeast exact are added to the process twice.

The process for producing a natural cheese of present claim 6 requires that the yeast exact are added to the process twice.

It is respectfully submitted that Gardiner in view of R2, Kimura and/or Germond, does not disclose or render obvious the claimed process for producing a natural cheese, as recited in present claim 6.

Conclusion

In view of the amendment to claim 6 and the foregoing remarks, Applicants respectfully submit that the present claims are not obvious over Gardiner, in view of R2, Kimura and/or Germond. Reconsideration and withdrawal of the present § 103(a) rejections of claims 6 and 9-10 are respectfully requested.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Short communication

Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp., *L. casei*, *L. paracasei* and *L. rhamnosus*) in cheddar cheese

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Abstract

Six batches of cheddar cheese were manufactured containing different combinations of commercially available probiotic cultures from three suppliers. Duplicate cheeses contained the organisms of each supplier, a *Bifidobacterium* spp. (each supplier), a *Lactobacillus acidophilus* (2 suppliers), and either *Lactobacillus casei*, *Lactobacillus paracasei*, or *Lactobacillus rhamnosus*. Using selective media, the different strains were assessed for viability during cheddar cheese maturation over 32 weeks. The *Bifidobacterium* sp. remained at high numbers with the three strains being present in cheese at 4×10^7, 1.4×10^8, and 5×10^8 CFU/g after 32 weeks. Similarly the *L. casei* (2×10^7 CFU/g), *L. paracasei* (1.6×10^7 CFU/g), and *L. rhamnosus* (9×10^8 CFU/g) strains survived well; however, the *L. acidophilus* strains performed poorly with both decreasing in a similar manner to be present at 3.6×10^3 CFU/g and 4.9×10^3 CFU/g after 32 weeks. This study indicates that cheddar cheese is a good vehicle for a variety of commercial probiotics but survival of *L. acidophilus* strains will need to be improved.

Keywords: Cheddar cheese; Probiotic; *Lactobacillus*; Bifodobacterium

1. Introduction

The recognition of cultured dairy products with probiotic bacteria as functional foods that provide health benefits beyond inherent basic nutrition and the emerging clinical evidence to their potential in preventing certain diseases has boosted their consumption (Playne et al., 2003; Boylston et al., 2004). Probiotic foods are currently restricted predominantly to fermented milk drinks and yogurt containing beneficial probiotic cultures such as lactobacilli and bifodobacterium, which are marketed as functional foods in Europe, Japan, USA and in Australia (Ross et al., 2002). A number of studies on the cultural aspects and the technology involved with fermented milks and yogurt as food carriers for probiotic bacteria have shown that these cultured products may not be optimal for the maintenance of recommended concentrations of some strains, as evidenced by poor viability in commercial yogurts (Rybka and Fleet, 1997; Gardiner et al., 1999; Vinderola et al., 2000; Shah et al., 2000). Cheese could be an alternate food vehicle to deliver viable probiotic bacteria in sufficient numbers to provide therapeutic benefit (Boylston et al., 2004). Incorporating a probiotic culture into a cheddar cheese would only produce a functional food if the culture remained viable in recommended numbers during maturation and shelf life of the product. It is also important that incorporation of probiotic bacteria does not affect the flavour, texture or appearance of a cheddar cheese (Mc Brearty et al., 2001). An alternate way to improve the survival of probiotic bacteria would be to incorporate them into a cheese where the pH, lipid content, oxygen level, and storage conditions are more conducive to the long-term survival of probiotic bacteria during cheese processing, maturation and shelf life (Boylston et al., 2004). In addition the matrix of the cheese, its high fat content and its high buffering capacity could offer protection to probiotic bacteria during passage through the gastrointestinal tract (Kailasapathy and Chin, 2000; Vinderola et al., 2002). However, in contrast to the short shelf life of probiotic fermented milks and yogurts, hard cheeses such as cheddar have long ripening period of up to 2 years, hence the development of probiotic cheese requires stringent selection of probiotic strains to maintain viability in the cheese throughout processing, maturation and storage period till consumption.

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Previous studies have shown that there are significant strain differences in the viability of probiotic bacteria during storage of cultured dairy products (Ross et al., 2002). When a number of commercially available probiotic products sold for human consumption were analysed, the identity and the number of incorporated species did not always correspond to those declared on the labels (Shah, 2000; Hamilton-Miller and Shah, 2002; Temmerman et al., 2002; Coeuret et al., 2004). Therefore, a routine application of many of these probiotic cultures may pose problems associated with low viability during cheese fermentation, manufacture and storage. This study was undertaken to evaluate the viability of eight different commercially available probiotic strains in a cheddar cheese throughout cheese making, ripening and storage. In this study, selective media were used for enumerating the numbers of probiotic bacteria in a complex microbial population composed of starter lactic acid bacteria, and non starter lactic acid bacteria (NSLAB) present during cheese maturation.

2. Materials and methods

2.1. Supply of microbial cultures and chemicals

Probiotic cultures were obtained from three commercial suppliers in Australia. Supplier 1 (DSM Food Specialties, Australia Pty Ltd., MooreBank, NSW, Australia) provided *Lactobacillus acidophilus* strain (LAFTI L10), *Bifidobacterium lactis* strain (LAFTI B94) and *Lactobacillus paracasei* (LAFTI L26). Supplier 2 (Chr. Hansen, Bayswater, Victoria, Australia), provided *Lactobacillus acidophilus* strain (La5), *Lactobacillus casei* (Lc1) and *Bifidobacterium lactis* (Bb12). Supplier 3 (Danisco, Copenhagen, Denmark) provided a *Bifidobacterium* sp. (HOWARU Bifido DR10) and *Lactobacillus rhamnosus* (HOWARU Rhamnosus DR20). The cultures were provided in freeze-dried form. The storage and maintenance of the cultures was carried out as per the recommendation of the manufacturers. Cheddar cheese (frozen DVS) starter cultures were received from DSM (DSM Food Specialties, Australia Pty Ltd., MooreBank, NSW, Australia). All chemicals were from Sigma (Castle Hill, NSW, Australia).

2.2. Cheddar cheese making

Cheddar cheese was manufactured according to the method described by the Australian Society for Dairy Technology (1977) using pasteurised milk (72.5 °C, 15 s) with 2% mixed starter culture (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) and 0.25% (v/v) calf rennet. About 10 l of pasteurised milk was standardised to a casein/fat ratio of 0.70 using skim milk. Annato and calcium chloride solutions were added at a rate of 0.25% (v/v) each. The probiotic cultures were added along with the cheese starter cultures. Cheese manufacture was carried out in a 10-l water jacketed vat fitted with a variable speed agitator blade (Armfield FT 20 A, Ringwood, England). After milling the curd, salt (sodium chloride) was applied at a rate of 2.5% (w/w) to the curd. The curd was placed in cheesecloth in a 10-cm hoop and pressed with an 8 kg weight overnight. The cheeses were cut into 6 slices and packaged in cryovac film and kept in a cheese maturation room (9–10 °C) to ripen. At different time periods, individual slices were sampled for probiotic bacterial numbers. Six batches of cheese were produced with duplicate batches containing the probiotic cultures from each of the three suppliers. Thus, one pair of cheeses contained *L. acidophilus* strain (L10), *B. lactis* strain (B94) and *L. paracasei* (L26), another pair contained *L. acidophilus* strain (La5), *L. casei* (Lc1) and *B. lactis* (Bb12) and the other pair of cheeses contained *Bifidobacterium* sp. (DR10) and *L. rhamnosus* (DR20).

2.3. Assessing viability of commercial probiotic cultures for cheese making

To establish the inoculation rate of probiotic cultures for cheese making all the commercial probiotic pure cultures were assessed for viable cell counts. Sterile peptone water and non-selective media were used i.e. Reconstituted Clostridial Agar (RCA, Oxoid, Therbarton, Australia) pH 5.5 for *Bifidobacterium* sp., *L. casei* and *L. rhamnosus* cultures and de Mann Rogosa, and Sharpe Agar (MRS, Oxoid) pH 6.2 for *L. acidophilus* cultures. The plates were incubated at 37 °C anaerobically for 2 days. An inoculum level to give greater than 10^8 CFU/g of cheese was added to the milk based on the CFU/g of the freeze-dried cultures. Generally this was close to 1 g of freeze-dried cultures except for L10 where it was 10 g.

2.4. Enumeration of probiotic bacteria in cheddar cheese

Two grams of cheese sample were homogenised aseptically in a stomacher with 18 ml of warm (45 °C) sterile 2% tri-sodium citrate solution and 10-fold (10^2 – 10^6) serial dilutions were prepared. Whey samples were mixed and then serially diluted in 2% tri-sodium citrate and processed as per the cheese samples. The enumeration was carried out using spread plates with a 100-µl inoculum on selective media. Spread plates were used as this facilitated colony differentiation. The media tested for *L. acidophilus* strains was MRS agar with bromocresol green and clindamycin (MRSBC). RCA was prepared following the manufacturer's recipe with the pH of the agar adjusted to 6.2. Bromocresol green stock solution was prepared at 0.2% (w/v), autoclaved at 121 °C for 15 min and added at the rate of 20 ml/l to the autoclaved molten MRS agar base. Clindamycin stock solution, prepared by dissolving 5 mg in 100 ml distilled water, was filter-sterilized and added at the rate of 2 ml/l to the autoclaved molten MRS agar base.

The media tested for *Bifidobacterium* spp. was a medium based on RCA with the addition of aniline blue and dicloxacillin (RCAAD). Aniline blue (0.3 g/l) was added to the RCA agar base, the pH was adjusted to 7.1, and the agar was then sterilized. Dicloxacillin stock solution (0.2% w/v; and filter-sterilized) was added at the rate of 1 ml/l to the autoclaved molten agar before pouring the plates.

RCA with bromocresol green and vancomycin (RCABV) was used for enumerating *L. paracasei*, *L. casei* and *L.*

rhamnosus. The pH of the RCA agar base was adjusted to 5.5 prior to autoclaving and then bromocresol green stock 0.2% w/v (prepared as previously described) added at the rate of 20 ml/l. Vancomycin stock solution (2% w/v) was prepared with distilled water and filter-sterilized through a 0.45-μm membrane. This was added at the rate of 0.5 ml/l to the molten agar. The plates were incubated anaerobically in gas jars using the GasPak System, (Oxoid) for 48 h at 37 °C prior to observation. All plate counts were carried out in duplicates. Plates containing 25–250 colonies were enumerated and recorded as colony forming units (CFU/g) of the product.

3. Results and discussion

Monitoring the viability of 8 probiotics strains in cheddar cheese over 32 weeks has indicated trends that are related to the different species of organism tested (Figs. 1–3). When the survival of the *Bifidobacterium* strains in duplicate cheeses was studied, it was seen that in the first 4 weeks, B94 showed an initial drop in numbers, whereas DR10 was unchanged and Bb12 numbers increased (Fig. 1). Subsequently the three strains showed similar trends with all increasing in numbers reaching a maximum at 12 weeks and then in the period 12 to 32 weeks all declined by a small amount. Thus B94, Bb12 and DR10 were 4×10^7 CFU/g, 1.4×10^8 CFU/g and 5×10^8 CFU/g, respectively (Fig. 1). Given a consumption of a nominal one serving (30 g) of cheese/day, the intake of each *Bifidobacterium* would be between 10^9 and 10^{10}/day that is well above the levels suggested as providing therapeutic benefits (Boylston et al., 2004). The viability of the *Bifidobacterium* spp. is similar to the results obtained by Dinakar and Mistry (1994) who found a *Bifidobacterium* sp. able to survive at 2×10^7 CFU/g after 24 weeks in cheddar cheese. Similarly Mc Brearty et al. (2001) found one isolate, *B. lactis* Bb-12 survived well in cheddar cheese at over 10^8 CFU/g; however, *Bifidobacterium longum* BB536 lost viability dropping to 10^5 CFU/g over a 6-month period.

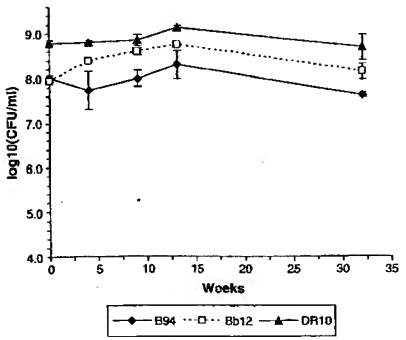

Fig. 1. Survival of three *Bifidobacterium* strains B94, Bb12, DR10 in six cheddar cheeses. The data is averaged from duplicate samples from two cheeses per strain. The error bars show standard deviations ($n=4$).

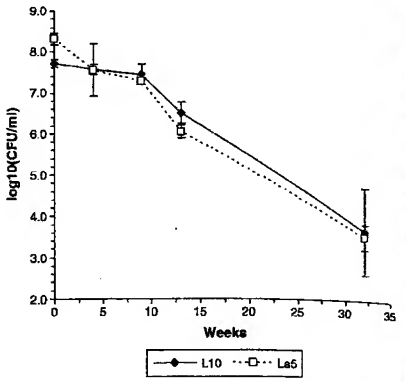

Fig. 2. Survival of two *L. acidophilus* strains L10 and La5 in four cheddar cheeses. The data is averaged from duplicate samples from two cheeses per strain The error bars show standard deviations ($n=4$).

The two *L. acidophilus* strains tested both demonstrated a different pattern of survival in cheddar cheese compared to the *Bifidobacterium* spp. One strain (L10) remained at a stable level for 8 weeks before dropping rapidly to reach 4.9×10^3 CFU/g after 32 weeks. The other strain, La5, started from a higher initial count and decreased from the first sampling at a rate very similar to L10 and dropped to a final population of 3.6×10^3 CFU/g (Fig. 2). The results previously reported with *L. acidophilus* strains used in cheese making have been variable though this may be influenced by the type of cheese and the probiotic strain studied. Godward and Kailasapathy (2003) reported that there was a decrease in cell numbers (approximately 2–3 log) of *L. acidophilus* CSCC 2401 and *L.*

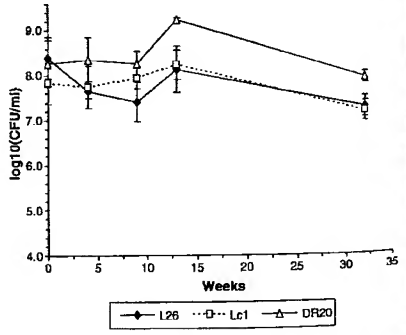

Fig 3. Survival of three *Lactobacillus* strains L26, Lc1, DR20 in six cheddar cheeses. The data is averaged from duplicate samples from two cheeses per strain (L26: *Lactobacillus paracasei*, Lc1: *Lactobacillus casei*. DR20: *Lactobacillus rhamnosus*). The error bars show standard deviations ($n=4$).

acidophilus 910 in cheddar cheese over a 24-week maturation period. With Gouda cheese, Gomes et al. (1995) found that there was an initial increase in *L. acidophilus* during manufacture but then there was a 2-log decrease in 9 weeks. This rate of decline is very similar, if slightly greater, to the rate of decline seen with our cultures in cheddar. A number of other reports indicate better survival of *L .acidophilus* but these tend to be young or non-ripened cheeses (Boylston et al., 2004).

The remaining *Lactobacillus* strains (L26, Lc1 and DR20) showed survival patterns similar to the *Bifidobacterium* spp. (Fig. 3). The *L. casei* (Lc1) maintained at the initial levels and then decreased slightly by 32 weeks to be present at 1.6×10^7 CFU/g. The *L. paracasei* (L26) lost viability up to 8 weeks, increased substantially to a maximum at 12 weeks, and then followed by a decline reaching 2.0×10^7 CFU/g by 32 weeks. The *L. rhamnosus* (DR20) followed a similar trend although it did not decrease initially and it increased more substantially before decreasing to 9×10^8 CFU/g at 32 weeks. It is possible that some of the colonies identified as probiotic may be endogenous non-starter lactic acid bacteria (NSLAB) though Crow et al. (2001) found these at lower numbers (maximum, 10^7 CFU/g) in New Zealand Cheddar. It was noticed in one of the cheeses that, after 32 weeks, there were two colony types on the RCABV media, although these had the same morphology on gram stain. Further confirmation of the exogenous and endogenous population may need to be done using RAPD PCR analysis of the NSLAB population as demonstrated by Mc Brearty et al. (2001).

It is interesting that all the probiotic strains tested except the *L. acidophilus* strains increased and reached a maximum population after 12 weeks. This period corresponds with the appearance of NSLAB in the cheese with this population reaching maximum levels in cheddar cheese around 8–12 weeks (Fitzsimmons et al., 2001; Crow et al., 2001). Two of the most commonly isolated NSLAB are *L. paracasei* and *L. rhamnosus* (Fitzsimmons et al., 2001) so the proliferation of the corresponding probiotics is not surprising. The decline in the numbers of *L. acidophilus* over this period may be similar to the decline seen with SLAB as the NSLAB increase (Stanton et al., 1998).

In other fermented products such as yogurts, the approach of adding a number of different probiotics using the A, B, C approach (for Acidophilus, Bifido, and Casei) has good recognition and acceptance by consumers. When marketing probiotic cheese, it may be desirable to extend the A, B, C approach to cheese but if the well-recognised *L. acidophilus* is to be included, its survival in cheese will need to be substantially improved. A better understanding of the reasons for this organism's inability to persist as cheese ripens would help define changes needed in manufacture. Alternately, existing approaches such as microencapsulation may offer improvements in survival (Chandramouli et al., 2004).

4. Conclusions

This study has demonstrated that cheddar cheese is a good vehicle for the delivery of a variety of commercial probiotics as these cultures remained viable at levels above the recommended 10^6–10^7 CFU/g after 32 weeks. All *Bifidobacterium* sp. survived well, as did *L. casei*, *L. paracasei* and *L. rhamnosus*. In contrast, *L. acidophilus* performed poorly and was found to be at levels well below the levels recommended for probiotic activity. The major differences between the probiotics survival were related to species differences and there was little variance between different commercial strains of the same *Bifidobacterium* or *L. acidophilus*.

Acknowledgements

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